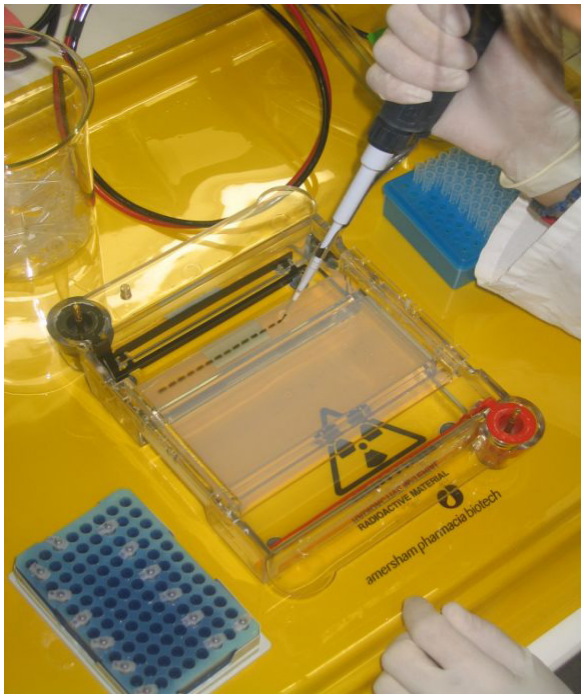
JRC Scientific and Technical Reports

Report on the Verification of the Performance of a MON 810 Event-specific Method on Maize Line MON 810 Using Real-time PCR

Validation Report and Protocol

M. Mazzara, E. Grazioli, C. Savini, G. Van den Eede



EUR 24237 EN-2009

JRC EUROPEAN COMMISSION

ihcp Institute for Health and Consumer Protection

Community Reference Laboratory CRL GM Food and Feed

The mission of the JRC-IHCP is to protect the interests and health of the consumer in the framework of EU legislation on chemicals, food, and consumer products by providing scientific and technical support including risk-benefit assessment and analysis of traceability.

European Commission
Joint Research Centre
Institute for Health and Consumer Protection

Contact information
Address: Molecular Biology and Genomics Unit
E-mail: JRC-BGMO@ec.europa.eu
Tel.: +39 0332 789379
Fax: +39 0332 786159

http://ihcp.jrc.ec.europa.eu/
http://www.jrc.ec.europa.eu/

JRC56609

EUR 24237 EN
ISBN 978-92-79-14982-5
ISSN 1018-5593
DOI 10.2788/59036

Luxembourg: Office for Official Publications of the European Communities

Printed in Italy

Report on the Verification of the Performance of a MON810 Event-specific Method on Maize Line MON810 Using Real-time PCR

28 May 2009

Joint Research Centre
Institute for Health and Consumer Protection
Molecular Biology and Genomics Unit

Executive Summary

The JRC as Community Reference Laboratory for GM Food and Feed (CRL-GMFF) (see Regulation EC No 1829/2003), has carried out an in-house verification study to assess the performance of the MON810 method to detect and quantify the MON810 transformation event in maize DNA (unique identifier MON-∅∅810-6). The method has previously undergone a full validation on samples represented by certified reference material. The present verification was conducted in order to verify the performance of the validated method on the control samples provided by the applicant as requested by Annex I.2.C.2 to Regulation (EC) No 641/2004 stating that "The method shall be applicable to samples of the food or feed, to the control samples and to the reference material, which is referred to in Articles 5(3)(j) and 17(3)(j) of Regulation (EC) No 1829/2003." The study was conducted according to internationally accepted guidelines [1,2].

In accordance with Regulation (EC) No 1829/2003 of 22 September 2003 on genetically modified food and feed and to Regulation (EC) No 641/2004 of 6 April 2004 on detailed rules for the implementation of Regulation (EC) No 1829/2003, the CRL-GMFF carried out a verification of the event-specific detection method previously validated by the Federal Institute for Risk Assessment (BfR) in collaboration with the American Association of Cereal Chemists (AACC), Joint Research Centre (JRC) of the European Commission (EC), Institute for Reference Material and Measurement (IRMM), the Institute for Health and Consumer Protection (IHCP) and GeneScan, Berlin; Monsanto Company provided the control samples (MON810 maize seeds and conventional maize seeds) used in the verification. The JRC prepared the in-house verification samples (calibration samples and blind samples at different GM percentages).

The results of the in-house verification study were evaluated with reference to ENGL method performance requirements (http://gmo-crl.jrc.ec.europa.eu/guidancedocs.htm) and to the results of the full validation (http://gmo-crl.jrc.ec.europa.eu/statusofdoss.htm).

The results of CRL-GMFF in-house verification study are made publicly available at http://gmo-crl.jrc.ec.europa.eu/statusofdoss.htm).

Drafted by:
C. Savini

Report Verification Team:
1) M. Ermolli

2) G. Pinski

Scientific and technical approval:
M. Mazzara

Compliance with CRL Quality System:
S. Cordeil

Authorisation to publish:
G. Van den Eede

Address of contact laboratory:
European Commission, Joint Research Centre
Institute for Health and Consumer Protection (IHCP)
Molecular Biology and Genomics Unit – Community Reference Laboratory for GM Food and Feed
Via Fermi 2749, I-21027 Ispra (VA)
ITALY

Report on Steps 1-3 of the Validation Process

The method for the event-specific detection of event MON810 in maize was developed and optimised by the Federal Institute for Risk Assessment (BfR). Monsanto Company submitted the control samples for the maize line containing event MON810 (unique identifier MON-∅∅810-6) under Articles 8 and 20 of Regulation (EC) No 1829/2003 of the European Parliament and of the Council "on genetically modified food and feed".

The Community Reference Laboratory for GM Food and Feed (CRL-GMFF), following reception of the documentation and material, including control samples, (step 1 of the validation process) carried out the scientific assessment of documentation and data (step 2) in accordance with Commission Regulation (EC) No 641/2004 "on detailed rules for the implementation of Regulation (EC) No 1829/2003 of the European Parliament and of the Council as regards the application for the authorisation of new genetically modified food and feed, the notification of existing products and adventitious or technically unavoidable presence of genetically modified material which has benefited from a favourable risk evaluation" and according to its operational procedures ("Description of the CRL-GMFF Validation Process", http://gmo-crl.jrc.ec.europa.eu/guidancedocs.htm).

The scientific assessment focussed on the method performance characteristics assessed against the method acceptance criteria set out by the European Network of GMO Laboratories and listed in the "Definition of Minimum Performance Requirements for Analytical Methods of GMO Testing", http://gmo-crl.jrc.ec.europa.eu/doc/Method%20requirements.pdf) (see Annex 1 for a summary of method acceptance criteria and method performance requirements).

The event-specific detection method for the maize line hosting the MON810 event was validated using certified reference material prepared by the Institute for Reference Materials and Measurements (IRMM). The CRL-GMFF performed an in-house verification of the detection method to verify the performance of the validated method on the control samples provided by the applicant as requested by Annex I.2.C.2 to Regulation (EC) No 641/2004 "The method shall be applicable to samples of the food or feed, to the control samples and to the reference material, which is referred to in Articles 5(3)(j) and 17(3)(j) of Regulation (EC) No 1829/2003."

In May 2009, the CRL-GMFF concluded the experimental verification of the method characteristics (step 3, experimental testing of the samples and methods) by quantifying five GM-levels within the range 0.10%-5.00% on a DNA mass basis. The experiments were performed under repeatability conditions and demonstrated that the PCR efficiency, linearity, trueness and repeatability of the quantification were within the limits established by the ENGL.

A Technical Report summarising the results of tests carried out by the CRL-GMFF (step 3) is available on request.

Content

1. Introduction

Monsanto Company submitted the control samples for maize event MON810 (unique identifier MON-∅∅810-6) in accordance with Articles 8 and 20 of Regulation (EC) No 1829/2003 of the European Parliament and of the Council "on genetically modified food and feed".

The Joint Research Centre (JRC, Molecular Biology and Genomics Unit of the Institute for Health and Consumer Protection) as Community Reference Laboratory for GM Food and Feed (see Regulation EC 1829/2003) carried out an in-house verification of the event-specific method for the detection and quantification of event MON810 maize. The method had been previously validated by an international collaborative trial (http://gmo-crl.jrc.ec.europa.eu/statusofdoss.htm) using a calibration sample and unknown samples consisting of certified reference material made of mixtures of genetically modified MON810 maize in conventional maize (w/w) between 0.1% and 5% (JRC, Institute for Reference Material and Measurement).

Upon reception of the method, samples and related data (step 1), the CRL-GMFF carried out the assessment of the documentation (step 2) and the in-house evaluation of the method (step 3), according to the requirements of Regulation (EC) 641/2004 and following its operational procedures. The in-house method verification was carried out in May 2009.

The operational procedure of the in-house verification included the following module:

- ✓ A method for DNA extraction from MON810 seeds, submitted by the applicant; the protocol for DNA extraction is available at http://gmo-crl.jrc.ec.europa.eu/statusofdoss.htm.
- ✓ Quantitative real-time PCR (Polymerase Chain Reaction). The methodology consists of an event-specific real-time quantitative TaqMan® PCR procedure for the determination of the relative content of event MON810 DNA to total maize DNA. The procedure is a simplex system, in which a maize *hmg* (high mobility group) endogenous assay (reference gene) and the target assay (MON810) are performed in separate wells.

The study was carried out in accordance with the following internationally accepted guidelines:

- ✓ ISO 5725:1994 [(1)].
- ✓ The IUPAC "Protocol for the design, conduct and interpretation of method-performance studies" [(2)].

2. Materials

For the verification of the quantitative event-specific method, control samples consisting of: whole maize seed heterozygous for MON810 (Lot Number GLP-0403-14800-S) and whole conventional maize seed (Lot Number GLP-0307-14210-S) were provided by the applicant in accordance with the provisions of Regulation (EC) No 1829/2003, Art 2.11 ["control sample defined as the GMO or its genetic material (positive sample) and the parental organism or its genetic material that has been used for the purpose of the genetic modification (negative sample)]. Genomic DNA was extracted from the control samples according to the procedure described in the Validated Method for MON810 (http://gmo-crl.jrc.ec.europa.eu/statusofdoss.htm).

Samples containing mixtures of MON810 and non-GM maize genomic DNA at different GMO concentrations were prepared by the CRL-GMFF, using the DNA extracted from the control samples provided, in a constant amount of total maize DNA.

The PCR protocol (reagents, concentrations, primer/probe sequences, amplification profile) followed in the in-house verification are as those already published as validated method for the validation of MON810.

Table 1 shows the five GM levels used in the verification of the MON810 detection method.

Table 1. MON810 GM contents

MON810 GM% (ng/ng DNA x 100)
0.10
0.50
1.00
2.00
5.00

3. Experimental design

Eight runs using the Mon810 method were carried out. In each run, samples were analysed in parallel with both the GM-specific system and the *hmg* reference system. Five GM-levels per run were examined and two replicates for each GM level were analysed. PCR analysis was performed in triplicate for all samples. In total, quantification of the five GM levels was performed as an average of sixteen replicate samples per GM level. An Excel spreadsheet was used for determination of GM%.

4. Method

Description of operational steps followed

For the specific detection of event MON810, a 92-bp fragment of the integration region of the construct inserted into the plant genome is amplified using two specific primers. PCR products are measured at each cycle (real-time) by means of a target-specific oligonucleotide probe labelled with two fluorescent dyes: FAM is used as the reporter dye at its 5′ end and TAMRA as a quencher dye at its 3′ end.

For the relative quantification of maize event MON810, a maize-specific reference system amplifies a 79-bp fragment of the maize endogenous gene *hmg* (*high mobility group*), using a pair of *hmg* gene-specific primers and an *hmg* gene-specific probe labelled with FAM and TAMRA.

For relative quantification of event MON810 in a DNA test sample, standard curves are generated both for the MON810 and the *hmg* reference systems by plotting the Ct values measured for the calibration samples against the logarithm of the DNA copy number and by fitting a regression line into these data. Thereafter, the standard curves are used to estimate the copy numbers in the unknown sample by interpolation from the standard curves.

For the determination of the amount of MON810 DNA in the unknown sample, the MON810 quantity is divided by the maize reference gene *hmg* quantity and multiplied by 100 to obtain the percentage value (GM% = GM-specific system/maize reference system x 100).

For detailed information on the preparation of standard curve calibration samples please refer to the protocol of the validated method at http://gmo-crl.jrc.ec.europa.eu/statusofdoss.htm.

5. Deviations reported

No deviations from the protocol of the two previously validated method were introduced.

6. Summary of results

PCR efficiency and linearity

The values of the slopes of the standard curves, from which the PCR efficiency is calculated using the formula [10^(-1/slope)-1]*100, and of the R^2 (expressing the linearity of the regression) are reported in Table 2.

Table 2. Values of standard curve slope, PCR efficiency and linearity (R^2) for the MON810 detection method (MON810 assay and endogenous *hmg* assay) on MON810 control samples

Run	MON810			*hmg*		
	Slope	PCR Efficiency (%)	Linearity (R^2)	Slope	PCR Efficiency (%)	Linearity (R^2)
1	-3.218	105	0.985	-3.308	101	0.995
2	-3.400	97	0.985	-3.252	103	0.997
3	-3.171	107	0.984	-3.305	101	0.997
4	-3.316	100	0.985	-3.151	108	0.998
5	-3.445	95	0.992	-3.240	104	0.997
6	-3.190	106	0.989	-3.304	101	0.997
7	-3.504	93	0.981	-3.248	103	0.997
8	-3.345	99	0.983	-3.199	105	0.996
Mean	-3.324	100	0.985	-3.251	103	0.997

The mean PCR efficiency of the GM and of the reference specific system was 100% and 103%, respectively. The linearity of the method was close to 0.99 for the GM-specific assay and to 1 for the reference specific assay. Data reported in Table 2 confirm the appropriate performance characteristics of the method tested on control samples.

7. Method performance requirements

The results of the in-house verification study for the MON810 detection method on control sample material are reported in Table 3. The results are evaluated with respect to the method acceptance criteria, as established by ENGL and adopted by CRL-GMFF (http://gmo-crl.jrc.ec.europa.eu/guidancedocs.htm, see also Annex 1). Further, Table 3 details estimates of accuracy and precision for each GM level.

Table 3. Trueness (expressed as bias %) and repeatability standard deviation (%) of the MON810 detection method on control samples of MON810.

MON810					
Unknown sample GM%	**Expected value (GMO %)**				
	0.1	**0.5**	**1.0**	**2.0**	**5.0**
Mean	**0.09**	**0.46**	**0.95**	**1.86**	**4.60**
SD	0.02	0.10	0.07	0.20	0.51
RSDr (%)	19.08	22.16	7.50	10.67	11.11
Bias %	-14	-9	-5	-7	-8

The *trueness* of the method is estimated using the measures of the method bias for each GM level. According to the ENGL acceptance criteria and method performance requirements, the accuracy of the quantification, measured as bias from the accepted value, should be ± 25% across the entire dynamic range. As shown in Table 3, the method satisfies the above requirement throughout its dynamic range.

Table 3 further documents the *relative repeatability standard deviation (*RSD_r*)* as estimated for each GM level. In order to accept methods for collaborative ring trial evaluation, the CRL-GMFF requires that RSD_r values be below 25%, as indicated by ENGL (Definition of Minimum Performance Requirements for Analytical Methods of GMO Testing" [http://gmo-crl.jrc.ec.europa.eu/guidancedocs.htm]).

As can be observed from the values reported in Table 3, the method satisfies this requirement across its dynamic range.

8. Comparison of the method performance between the verification and the full validation

A synoptic comparison of the method performance as assessed through the ring-trial carried out on certified reference material and the present verification on control samples is provided in Table 4.

Table 4. Comparison of trueness (bias %) and repeatability standard deviation (%) of the MON810 detection method assessed through in-house verification on control samples and full validation on certified reference materials (CRM)

Trueness and repeatability of MON810 quantification on MON810 control samples			**Trueness and repeatability of MON810 quantification on CRM***		
GM%	**Bias (%)**	**RSDr (%)**	**GM%**	**Bias (%)**	**RSDr (%)**
0.1	-14	19	**0.10**	2.3	36
0.5	-9	22	**0.50**	-7.7	21
1.0	-5	7.5	**1.00**	-17	17
2.0	-7	11	**2.00**	-11	16
5.0	-8	11	**5.00**	-9.7	29

* validated method (http://gmo-crl.jrc.ec.europa.eu/statusofdoss.htm)

In terms of repeatability, when applied to the control samples, the MON810 detection method shows lower RSDr (%) for most of the GM levels, compared to the validation results obtained on certified reference material. In terms of trueness, the method verification provided comparable or lower bias (%) across the GM levels in comparison to the bias (%) obtained in the full validation, with the exception of a bias of 14% at the GM level 0.1%.

Therefore, the in-house method verification has demonstrated that the MON810 method can be equally applied for the quantification of the MON810 event in control samples.

9. Conclusions

The overall method performance of the method for the quantitative detection of event MON810 has been evaluated with respect to the method acceptance criteria and the method performance requirements recommended by the ENGL (as detailed at http://gmo-crl.jrc.ec.europa.eu/guidancedocs.htm), and to the full validation results obtained on certified reference materials (http://gmo-crl.jrc.ec.europa.eu/statusofdoss.htm).

The results of the present verification study indicate that the analytical modules comply with the ENGL performance criteria. The method is therefore applicable to the control samples provided (see paragraph 3 "Materials"), in accordance with the requirements of Annex I-2.C.2 to Commission Regulation (EC) No 641/2004.

10. Quality assurance

The CRL-GMFF carries out all operations according to ISO 9001:2000 (certificate number: CH-32232) and ISO 17025:2005 (certificate number: DAC-PL-0459-06-00) [DNA extraction, qualitative and quantitative PCR in the area of Biology (DNA extraction and PCR method validation for the detection and identification of GMOs in food and feed materials)]

11. References

1. Horwitz, W., 1995. Protocol for the design, conduct and interpretation of method performance studies, *Pure and Appl. Chem*, 67, 331-343.
2. International Standard (ISO) 5725, 1994. Accuracy (trueness and precision) of measurement methods and results. International Organization for Standardization, Genève, Swizerland.

12. Annex 1: method acceptance criteria and method performance requirements as set by the European Network of GMO Laboratories (ENGL)

Method Acceptance Criteria should be fulfilled at the moment of submission of a method (Phase 1: acceptance for the collaborative study).

Method Performance Requirements should be fulfilled in a collaborative study in order to consider the method as fit for its purpose (Phase 2: evaluation of the collaborative study results).

Method Acceptance Criteria

Applicability

Definition: The description of analytes, matrices, and concentrations to which a method can be applied.

Acceptance Criterion: The applicability statement should provide information on the scope of the method and include data for the indices listed below for the product/s for which the application is submitted. The description should also include warnings to known interferences by other analytes, or inapplicability to certain matrices and situations.

Practicability

Definition: The ease of operations, the feasibility and efficiency of implementation, the associated unitary costs (e.g. Euro/sample) of the method.

Acceptance Criterion: The practicability statement should provide indication on the required equipment for the application of the method with regards to the analysis *per se* and the sample preparation. An indication of costs, timing, practical difficulties and any other factor that could be of importance for the operators should be indicated.

Specificity

Definition: Property of a method to respond exclusively to the characteristic or analyte of interest.

Acceptance Criterion: The method should be event-specific and be functional only with the GMO or GM based product for which it was developed. This should be demonstrated by empirical results from testing the method with non-target transgenic events and non-transgenic material. This testing should include closely related events and cases where the limit of the detection is tested.

Dynamic Range

Definition: The range of concentrations over which the method performs in a linear manner with an acceptable level of accuracy and precision.

Acceptance Criterion: The dynamic range of the method should include the 1/10 and at least 5 times the target concentration. Target concentration is intended as the threshold relevant for legislative requirements. The acceptable level of accuracy and precision are described below. The range of the standard curve(s) should allow testing of blind samples throughout the entire dynamic range, including the lower (10%) and upper (500%) end.

Accuracy

Definition: The closeness of agreement between a test result and the accepted reference value.

Acceptance Criterion: The accuracy should be within ± 25% of the accepted reference value over the whole dynamic range.

Amplification Efficiency

Definition: The rate of amplification that leads to a theoretical slope of –3.32 with an efficiency of 100% in each cycle. The efficiency of the reaction can be calculated by the following equation: Efficiency = $[10^{(-1/slope)}] - 1$

Acceptance Criterion: The average value of the slope of the standard curve should be in the range of (- 3.1 ≥ slope ≥ - 3.6)

R^2 Coefficient

Definition: The R^2 coefficient is the correlation coefficient of a standard curve obtained by linear regression analysis.

Acceptance Criterion: The average value of R^2 should be ≥ 0.98.

Repeatability Standard Deviation (RSD_r)

Definition: The standard deviation of test results obtained under repeatability conditions. Repeatability conditions are conditions where test results are obtained with the same method, on identical test items, in the same laboratory, by the same operator, using the same equipment within short intervals of time.

Acceptance Criterion: The relative repeatability standard deviation should be below 25% over the whole dynamic range of the method.

Note: Estimates of repeatability submitted by the applicant should be obtained on a sufficient number of test results, at least 15, as indicated in ISO 5725-3 (1994).

Limit of Quantitation (LOQ)

Definition: The limit of quantitation is the lowest amount or concentration of analyte in a sample that can be reliably quantified with an acceptable level of precision and accuracy.

Acceptance Criterion: LOQ should be less than $1/10^{th}$ of the value of the target concentration with an RSD_r ≤ 25%. Target concentration should be intended as the threshold relevant for legislative requirements. The acceptable level of accuracy and precision are described below.

Limit of Detection (LOD)

Definition: The limit of detection is the lowest amount or concentration of analyte in a sample, which can be reliably detected, but not necessarily quantified, as demonstrated by single laboratory validation.

Acceptance Criterion: LOD should be less than $1/20^{th}$ of the target concentration. Experimentally, quantitative methods should detect the presence of the analyte at least 95% of the time at the LOD, ensuring $\leq$ 5% false negative results. Target concentration should be intended as the threshold relevant for legislative requirements.

Robustness

Definition: The robustness of a method is a measure of its capacity to remain unaffected by small, but deliberate deviations from the experimental conditions described in the procedure.

Acceptance Criterion: The response of an assay with respect to these small variations should not deviate more than $\pm$ 30%. Examples of factors that a robustness test could address are: use of different instrument type, operator, brand of reagents, concentration of reagents, and temperature of reaction.

Method Performance Requirements

Dynamic Range

Definition: In the collaborative trial the dynamic range is the range of concentrations over which the reproducibility and the trueness of the method are evaluated with respect to the requirements specified below.

Acceptance Criterion: The dynamic range of the method should include the 1/10 and at least five times the target concentration. Target concentration should be intended as the threshold relevant for legislative requirements.

Reproducibility Standard Deviation (RSD_R)

Definition: The standard deviation of test results obtained under reproducibility conditions. Reproducibility conditions are conditions where test results are obtained with the same method, on identical test items, in different laboratories, with different operators, using different equipment. Reproducibility standard deviation describes the inter-laboratory variation.

Acceptance Criterion: The relative reproducibility standard deviation should be below 35% at the target concentration and over the entire dynamic range. An RSD_R < 50 % is acceptable for concentrations below 0.2%.

Trueness

Definition: The closeness of agreement between the average value obtained from a large series of test results and an accepted reference value. The measure of trueness is usually expressed in terms of bias.

Acceptance Criterion: The trueness should be within $\pm$ 25% of the accepted reference value over the whole dynamic range.

EUROPEAN COMMISSION
DIRECTORATE GENERAL JRC
JOINT RESEARCH CENTRE
INSTITUTE FOR HEALTH AND CONSUMER PROTECTION
COMMUNITY REFERENCE LABORATORY FOR GM FOOD AND FEED

CRL assessment on the validation of an event specific method for the relative quantitation of maize line MON 810 DNA using real-time PCR as carried out by Federal Institute for Risk Assessment (BfR)

Biotechnology & GMOs Unit
Institute for Health and Consumer Protection
DG Joint Research Centre

10 March 2006

Executive Summary

An event-specific method for the quantitation of maize MON 810 by means of real-time PCR has been validated in a collaborative trial by the Federal Institute for Risk Assessment (BfR) in collaboration with the American Association of Cereal Chemists (AACC), Joint Research Centre (JRC) of the European Commission (EC), Institute for Reference Material and Measurement (IRMM), the Institute for Health and Consumer Protection (IHCP) and GeneScan, Berlin.

The trial involved fifteen laboratories and was conducted according to internationally accepted guidelines.

The method is annexed to the standard ISO 21570:2005, "Foodstuffs -- Methods of analysis for the detection of genetically modified organisms and derived products -- Quantitative nucleic acid based methods".

Contents

Document Approval		
Name / *Function*	**Date**	**Signature**
Marco Mazzara *Sector Head*	**10/03/2006**	**Signed**
Stephane Cordeil *Quality Manager*	**10/03/2006**	**Signed**
Guy Van den Eede *B&GMOs Unit Head*	**10/03/2006**	**Signed**

Address of contact laboratories:

Federal Institute for Risk Assessment
Bundesinstitut fuer Risikobewertung (BfR)
Dahlem
FG 213,
Thielallee 88-92,
D-14195 Berlin

European Commission, Joint Research Centre
Institute for Health and Consumer Protection (IHCP)
Biotechnology and GMOs Unit – Community Reference Laboratory
Via Fermi 1, 21020 Ispra (VA) - Italy

1. General information

This protocol describes an event-specific detection and quantitative TaqMan® PCR procedure for the relative determination of event MON 810 maize in total maize. The real time PCR was optimized for block thermal cycler. Template DNA extracted should be tested for quality and quantity prior to PCR assay.

For specific detection of event MON 810 maize a 92 bp fragment of the single copy DNA integration-border region of the genomic sequence and the inserted sequence element originating from CaMV (35S promoter) as a result of *in vitro* recombination present in the genetically modified insect-protected MON 810 ("YieldGuard") maize (Monsanto) is amplified in TaqMan® PCR.

For relative quantitation of MON 810 maize, a 79 bp fragment of the taxon specific maize (*Zea mays*) high mobility group protein gene (hmg) gene using a gene specific combination of primers and probe is amplified.

The measured fluorescence signal passes a threshold value after a certain number of cycles. This threshold cycle is called "Ct-value". For quantitation of the amount of event MON 810 maize in a test sample, event MON 810 and hmg Ct values are determined for the sample. A standard curve procedure is then used to calculate the relative number of MON 810 specific genome copies to total maize genome copies.

2. Validation status

The method was optimized for ground maize seeds (certified reference materials [CRM IRMM-413]), containing mixtures of genetically modified MON 810 and conventional maize.

The reproducibility and accuracy of the method was tested through a collaborative study using samples at different GMO contents.

The method was originally developed for the ABI PRISM® 7700 Sequence Detection System (SDS).

This method has been validated in a collaborative study conducted by the Federal Institute for Risk assessment (BfR) in collaboration with The American Association of Cereal Chemists (AACC), Joint Research Centre (JRC) of the European Commission (EC), Institute for Reference Material and Measurement (IRMM) and Institute for Health and Consumer Protection (IHCP) and GeneScan.

The operational procedure of the collaborative study comprised the following modules:

- DNA extraction: GENESpin DNA extraction system (GeneScan)

- Quantitative real-time PCR (Polymerase Chain Reaction): for detection of event MON 810 maize a 92 bp fragment of the single copy DNA integration-border region of the genomic sequence and the inserted sequence element originating from CaMV (35S promoter) as a result of *in vitro* recombination present in the genetically modified insect-protected MON 810 ("YieldGuard") maize (Monsanto) was amplified in TaqMan® PCR

The ring-trial was carried out in accordance with the following internationally accepted guidelines:

- ISO 5725 (1994).
- The IUPAC "Protocol for the design, conduct and interpretation of method performance studies" (Horwitz, 1995).

The study was undertaken with 15 laboratories using either the ABI PRISM® 7700, ABI PRISM® 7900 (Applied Biosystems Inc) or the iCycler iQ Real-Time PCR Detection System (Bio-Rad Laboratories).

Fourteen laboratories from countries all over the world reported results.

For each unknown sample one DNA extraction has been carried out. Each test sample was analyzed by real time PCR in 3 repetitions.
Each participant received 12 unknown samples. The samples consisted of 6 certified reference materials (CRM IRMM-413) between <0,02 % and 5 % GM MON 810 in conventional maize (w/w).

Each laboratory received each level of GM MON 810 CRM in two separate unknown samples.
Details of the results of the collaborative study performed in 2003/2004 are shown in table 1.

Table 1: Statistics of the collaborative study for the real time PCR procedure to quantify MON 810 specific material.

Sample	Sample 1 <0.02 %	Sample 2 0.1 %	Sample 3 0.5 %	Sample 4 1 %	Sample 5 2%	Sample 6 5 %
Number of laboratories reported	11	14	14	14	14	14
Number of outliers	1	1	0	2	0	0
Number of laboratories retained after eliminating outliers	10	13	14	12	14	14
Mean value (%)	0.028	0.1023	0.4613	0.8327	1.7814	4.5154
Repeatability standard deviation s_r	0.00736	0.03641	0.9606	0.13744	0.28385	1.29374
Repeatability relative standard deviation RSD_r (%)	26.27	35.60	20.82	16.51	15.93	28.65
Repeatability limit r (r = 2,8 x s_r)	0.0206	0.1019	0.269	0.3848	0.7948	3.6225
Reproducibility standard deviation s_R	0.02326	0.04646	0.20068	0.26534	0.56609	1.65451
Reproducibility relative standard deviation RSD_R (%)	83.03	45.43	43.5	31.86	31.78	36.64
Reproducibility limit R (R=2,8 x s_R)	0.0651	0.1301	0.5619	0.743	1.5851	4.6326
Bias (%)	-	2.3	- 7.74	- 16.73	- 10.93	- 9.69

a Outliers were identified with the Grubbs and Cochran tests

These results are evaluated with respect to the method acceptance criteria and to the method performance requirements, as established by ENGL and adopted by CRL. In table 1, estimates of both repeatability and reproducibility for each GM level are reported, after identification and removal of outliers through Cochran and Grubbs tests, according to ISO 5725-2.

Method bias, which allows estimating trueness, is reported for each GM level in table 1. Bias is estimated according to ISO 5725 data analysis protocol. According to ENGL method performance requirements, trueness should be □25% throughout the whole dynamic range. The method satisfies such requirement for all GM values tested.

The relative reproducibility standard deviation (RSDR), that describes the inter-laboratory variation, should be below 33% at the target concentration and over the majority of the dynamic range, while it should be below 50% at the lower end of the dynamic range. As it can be observed in table 1, the method satisfies this requirement at the target concentration (1%) and at GM level of 0.1% and 2%; a minor deviation can be seen at 5% (36.64), while the RSDR at GM level 0.5% is 43.5

3. Specificity

Specificity tests prior to the study showed no cross reactivity of the detection systems to the following non-target species/samples: soybean DNA.

No cross reactivity has been occurred with the following genetically modified maize: Event176, Bt11, T25, GA21 and GTS 40-3-2 soybean.

4. Limit of detection (LOD)

According to the method developer, the absolute LOD has been determined to be 5 copies of the target sequence

According to the method developer, the relative LOD has been demonstrated to be at least 0.1 % (w/w).

5. Limit of quantitation (LOQ)

According to the method developer, the absolute limit of quantitation has been determined to be 10 copies of the target sequence.

According to the method developer the relative limit of quantitation has been determined to be at least 0.1% (equal to the lowest concentration point of the calibration curve used; [w/w]).

6. Procedure

All handling of reagents and controls should occur in an ISO 17025 environment or equivalent.

Further appropriate ISO/EN Norms dealing with the detection and quantitation of GMO derived material should be taken into consideration.

7. Primer/probe systems

The following primers and TaqMan® probes were used in the collaborative study (table 2).

Table 2 : Primer and probe sequences

Name	Oligonucleotide DNA sequence	Final conc. in PCR
Reference gene target sequence		
ZM1-F	5´-TTg gAC TAg AAA TCT CgT gCT gA-3‘	300 nmol/l
ZM1-R	5´-gCT ACA TAg ggA gCC TTg TCC T-3‘	300 nmol/l
Probe ZM1	5´-FAM—CAA TCC ACA CAA ACg CAC gCg TA-TAMRA-3‘	160 nmol/l
GMO target sequence		
Mail-F1	5´-TCg AAg gAC gAA ggA CTC TAA CgT-3´	300 nmol/l
Mail-R1	5´-gCC ACC TTC CTT TTC CAC TAT CTT-3´	300 nmol/l
Probe Mail-S2	5´-FAM-AAC ATC CTT TgC CAT TgC CCA gC-TAMRA P-3´	180 nmol/l
FAM: 6-carboxylfluorecein, TAMRA: 6-carboxytetramethylrhodamine		

8. Sample extraction

For DNA extraction the GENESpin DNA extraction system (GeneScan) was used according to the manufacturer’s instruction.

9. PCR set-up

The PCR set-up for the taxon specific target sequence and for the GMO target sequence should be carried out in separate vials. Multiplex PCR (using differential fluorescent labels for the probes) has not been tested or validated.

The method is described for a total volume of 25 µl per reaction mixture with the reagents listed in Table 3.

Table 3: Amplification reaction mixture in the final volume/concentration per reaction vial

Total reaction volume		25 µl
Template DNA added (2,3 ng to 150 ng maize DNA)		5 µl
DNA polymerase	AmpliTaq Gold® (Applied Biosystems Inc)	1.25 U
Decontaminati on system	dUTP AmpErase uracil N-glycosylase	400 µmol/l 0.5 U
Reaction buffer	TaqMan™ buffer A	1 fold
	$MgCl_2$	6.5 mmol/l
Primers	see Table D.6	see Table 2
DNTP	dATP. dCTP. dGTP	200 µmol/l each
Probe	see Table 2	see Table 2

As a positive control and as calibrant reference material, certified reference materials of MON810 (material containing <0.02 % to 5 % of genetically modified maize) produced by IRMM, Geel, Belgium (IRMM-413 series) may be used.

A series of 1:4 dilution steps of DNA from 5 % CRM is used to establish the standard curves for the MON 810 specific and hmg specific PCR, respectively.

10. Temperature-time-programme

The temperature-time-programme as outlined in Table 4 was optimised for the ABI PRISM® 7700 Sequence Detection System (Applied Biosystems Inc). In the validation study it was used in combination with the AmpliTaq Gold® DNA polymerase. Table 4 describes the reaction conditions.

Table 4: Procedure - Reaction conditions

		Time (s)	**Temperature (°C)**
Pre-PCR – decontamination (optional)		120	50
Pre-PCR – activation of DNA polymerase and denaturation of template DNA		600	95
PCR (45 cycles)			
	Denaturation	15	95
	Annealing Elongation	60	60

11. Data analysis

The baseline range is usually set to cycles 3 to 15. If amplifications do not appear before cycle 20, the baseline stop can be extended to cycle 20.

After defining a threshold value within the logarithmic phase of amplification (e.g. 0.01 to 0.1 normalized reporter dye fluorescence [Rn]) the instruments software calculates the Ct values for each reaction. The Ct values measured for the calibration points in the taxon specific maize or MON 810 specific PCR system, respectively, are plotted against the natural logarithm

of the DNA copy numbers introduced into PCR. The copy numbers measured for the unknown sample DNA are obtained by interpolation from the standard curves.

A calibration curve is produced by plotting Ct values against the logarithm of the target copy number for the calibration points.

For the determination of the amount of MON 810 DNA in the test sample, the MON 810 copy number is divided by the number of maize genome equivalents and multiplied by 100 to get the percentage value.

12. Conclusions

The overall method performance has been evaluated with respect to the method acceptance criteria and method performance requirements recommended by the ENGL (available at http://gmo-crl.jrc.it).

The results obtained during the collaborative trial indicate that the method can be considered as fit for enforcement purposes with respect to its trueness and inter-laboratory variability, taking into account the observations on RSD_R reported above.

13. References

ISO 21570:2005, "Foodstuffs -- Methods of analysis for the detection of genetically modified organisms and derived products -- Quantitative nucleic acid based methods".

European Commission

EUR 24237 EN – Joint Research Centre – Institute for Health and Consumer Protection
Title: Report on the Verification of the Performance of a MON810 Event-specific Method on Maize Line MON810 Using Real-time PCR
Author(s): M. Mazzara, E. Grazioli, C. Savini, G. Van den Eede
Luxembourg: Office for Official Publications of the European Communities
2009 – 23 pp. – 21 x 29.7 cm
EUR – Scientific and Technical Research series – ISSN 1018-5593
ISBN 978-92-79-14982-5
DOI 10.2788/59036

Abstract

The JRC as Community Reference Laboratory for GM Food and Feed (CRL-GMFF) (see Regulation EC No 1829/2003), has carried out an in-house verification study to assess the performance of the MON810 method to detect and quantify the MON810 transformation event in maize DNA (unique identifier MON-∅∅810-6). The method has previously undergone a full validation on samples represented by certified reference material. The present verification was conducted in order to verify the performance of the validated method on the control samples provided by the applicant as requested by Annex I.2.C.2 to Regulation (EC) No 641/2004 stating that "The method shall be applicable to samples of the food or feed, to the control samples and to the reference material, which is referred to in Articles 5(3)(j) and 17(3)(j) of Regulation (EC) No 1829/2003." The study was conducted according to internationally accepted guidelines [(1,2)].

In accordance with Regulation (EC) No 1829/2003 of 22 September 2003 on genetically modified food and feed and to Regulation (EC) No 641/2004 of 6 April 2004 on detailed rules for the implementation of Regulation (EC) No 1829/2003, the CRL-GMFF carried out a verification of the event-specific detection method previously validated by the Federal Institute for Risk Assessment (BfR) in collaboration with the American Association of Cereal Chemists (AACC), Joint Research Centre (JRC) of the European Commission (EC), Institute for Reference Material and Measurement (IRMM), the Institute for Health and Consumer Protection (IHCP) and GeneScan, Berlin; Monsanto Company provided the control samples (MON810 maize seeds and conventional maize seeds) used in the verification. The JRC prepared the in-house verification samples (calibration samples and blind samples at different GM percentages).

The results of the in-house verification study were evaluated with reference to ENGL method performance requirements (http://gmo-crl.jrc.ec.europa.eu/guidancedocs.htm) and to the results of the full validation (http://gmo-crl.jrc.ec.europa.eu/statusofdoss.htm).

The results of CRL-GMFF in-house verification study are made publicly available at http://gmo-crl.jrc.ec.europa.eu/statusofdoss.htm).

The mission of the JRC is to provide customer-driven scientific and technical support for the conception, development, implementation and monitoring of EU policies. As a service of the European Commission, the JRC functions as a reference centre of science and technology for the Union. Close to the policy-making process, it serves the common interest of the Member States, while being independent of special interests, whether private or national.

JRC
EUROPEAN COMMISSION

LB-NA-24237-EN-C

Publications Office
Publications.europa.eu

ISBN 978-92-79-14982-5